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14. ABSTRACT

Spinal cord injury (SCI) exacts an enormous social and financial burden on society. As such, there has been considerable attention directed at finding treatment strategies, including development of tissue and cell transplant techniques. However, the current approaches do not adequately address the complexity of the injury site, such as lesion length and an environment that is usually non-permissive for axon regeneration. We have developed tissue engineered nerve grafts (TENGs) consisting of living dorsal root ganglia (DRG) and axons that can be stretch-grown to a length necessary to bridge extensive lesions. In current studies, we have optimized in vivo laminoplasty procedures, transplanted stretch-grown constructs into a 5 mm-long lesion in the rat thoracic spinal cord, and have demonstrated survival of DRGs up to 6 weeks post-transplant. We intend to evaluate long-term (3 and 6 month) survival of the constructs as well as functional recovery beyond the lesion site. If successful, this approach will provide an alternative or additional means to repair large spinal lesions.

15. SUBJECT TERMS

None provided

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INTRODUCTION

In 2013, the National Spinal Cord Injury Statistical Center (NSCISC) reported that there were approximately 40 cases per million population in the United States, or approximately 12,000 new cases, of spinal cord injury (SCI) each year[1]. In addition to these new cases, approximately 273,000 people in the United States live with a chronic SCI[1]. SCIs have become a common consequence of motor vehicle crashes, falls, and acts of violence (most typically gunshot wounds). Prior to receiving funding for this grant, our research group established proof-of-concept success of bridging a lateral hemisection in the rat spinal cord with engineered ("stretch-grown") living nervous tissue constructs referred to as TENGs (tissue engineered nerve grafts)[2]. However, that model is not useful for positively identifying host axons growing across the lesion or for functional assessment (the animals recover most hindlimb function).

For the current research effort, we initially developed a new model of a 1 cm long complete evacuation of the thoracic spinal cord. Notably, this model completely removes all tissue including axon tracts, which produces complete hindlimb paralysis. With this new model, we have initiated studies to 1) examine functional recovery following transplantion of the living constructs bridging the SCI lesion, and 2) determine potential formation of new intraspinal circuits across the lesion, such as growth of host axons through the construct and synapse formation with neurons on the other side. Our first year was dedicated to hiring and training new personnel, developing and characterizing a new model of SCI, and initiating our transplantation studies. In the second year, we improved upon the laminoplasty technique used by providing extradural protection that minimizes connective tissue infiltration and compression of the transplanted constructs. Using this new method, we observed improved neuronal survival and axonal projections out to 1 month post-transplant. However, we continued to see some compression and connective tissue infiltration. Therefore, this third year we modified the model to a 5 mm long complete evacuation. Like the 1 cm complete evacuation, a 5 mm long complete evacuation removes all spinal cord tissue and produces complete hindlimb paralysis, but a 5 mm long evacuation notably minimizes the invasiveness of the surgical procedure and provides more support to the TENGs as more native tissue is available surrounding the injury site. With this modified model, we have observed minimal connective tissue infiltration compared to the 1 cm model as well as the absence of compression without the need for a half-tube cover. In addition, we have introduced passive rehabilitation using motorized bikes and functional assessment using the Basso, Beattie, and Bresnahan (BBB) scale.

BODY

Specific Aim 1: Evaluation of effects of transplanted nervous tissue constructs on recovery of function over 3 months post-injury in a model of complete spinal cord segment excision (T9-T11).

We now transplant tissue engineered nerve grafts (TENGs) consisting of long axonal tracts (5 mm long) and two populations of dorsal root ganglia (DRG) to bridge an excised segment (also 5 mm in length) of the rat thoracic T10-T11 spinal cord. A primary injury is performed to remove the cord and fill the cavity with a collagen only hydrogel. The TENG is then transplanted during a secondary surgery, 9-10 days after the initial injury. During this surgery, the initial hydrogel is carefully removed and the TENG is transplanted within a new collagen hydrogel. Experimental groups include animals that receive the collagen only hydrogel during the secondary injury or animals that receive a TENG embedded in the collagen hydrogel. Furthermore, groups are divided into animals that do or do not receive passive rehabilitation. The passive rehabilitation is an exercise regimen using motorized bikes that is performed three times per week for 60 minute sessions (with a 10 minute break). We also perform weekly functional outcome assessments using the BBB scale to evaluate potential recovery of motor function.

Specific Aim 2: Evaluation of the survival and integration of transplanted living nervous tissue constructs and host axon regeneration through the construct at 1 month and 3 months post transplantation. Using the same animals/groups from Aim 1, we have performed extensive histological examinations on constructs at 10 days, 4 weeks, and 6 weeks post-transplant. As described in our 2012 Annual Report, we addressed earlier problems with connective tissue infiltration into the vertebral column and physical compression from the closure method by incorporating a more sufficient extradural barrier into our surgical procedure. This extradural barrier, which includes a layer of Teflon tape followed by a Gelfoam® sealed with Vetbond™ Tissue Adhesive, helped physically block connective tissue invasion and protect against compression. All TENGs are virally transduced to express green fluorescent protein (GFP) with an AAV viral vector (AAV2/1.hSynapsin.EGFP.WPRE.bGH, UPenn Vector Core) to permit *in vivo* identification.

Results: Our 5 mm long complete evacuation model (Figure 1) has been successful in minimizing connective tissue infiltration as well as preventing compression of the spinal cord and transplant without the need for a half-tube cover. TENGs are consistently robust and brightly fluorescent at the time of transplantion (Figure 1C). Additionally, TENGs remain viable even though animals do not receive any immunosupressants. This high TENG viability is observed at 10 days (Figures 3 and 4) and 6 weeks (Figure 6) post-transplant. Furthermore, our findings thus far provide preliminary evidence of host axon integration with and along TENG axons (Figures 4 and 6), a phenomenon our group has previously observed in peripheral nerve injuries treated with TENGs. (Please note, these findings need to be confirmed with clearer host-graft differentiation, since red SMI31/32 axons cannot definitively be shown to be host axons in this model. TENG axons will stain positively for SMI 31/32 also.) We have designed experiments using two different methods to more carefully identify host vs. graft. The first method is to enroll GFP+ host animals and to transplant mCherry-red transduced TENGs. In this manner, all host axons will fluoresce green only and all TENG axons will fluoresce red only. From histological analysis we will be able to discriminate one axon from the other. The second method we are using to differentiate between graft and host axons is to employ anterograde tracing of descending motor tracts prior to sacrifice. This will allow us to trace host axonal projections from their point of origin to their point of termination.

Preliminary analysis of scores gathered on the BBB scale has shown no significant differences between experimental groups thus far (Figure 2). We will continue to collect data weekly for this parameter out to the final time point of 12 weeks and will re-evaluate at that time for statistical differences amongst groups.

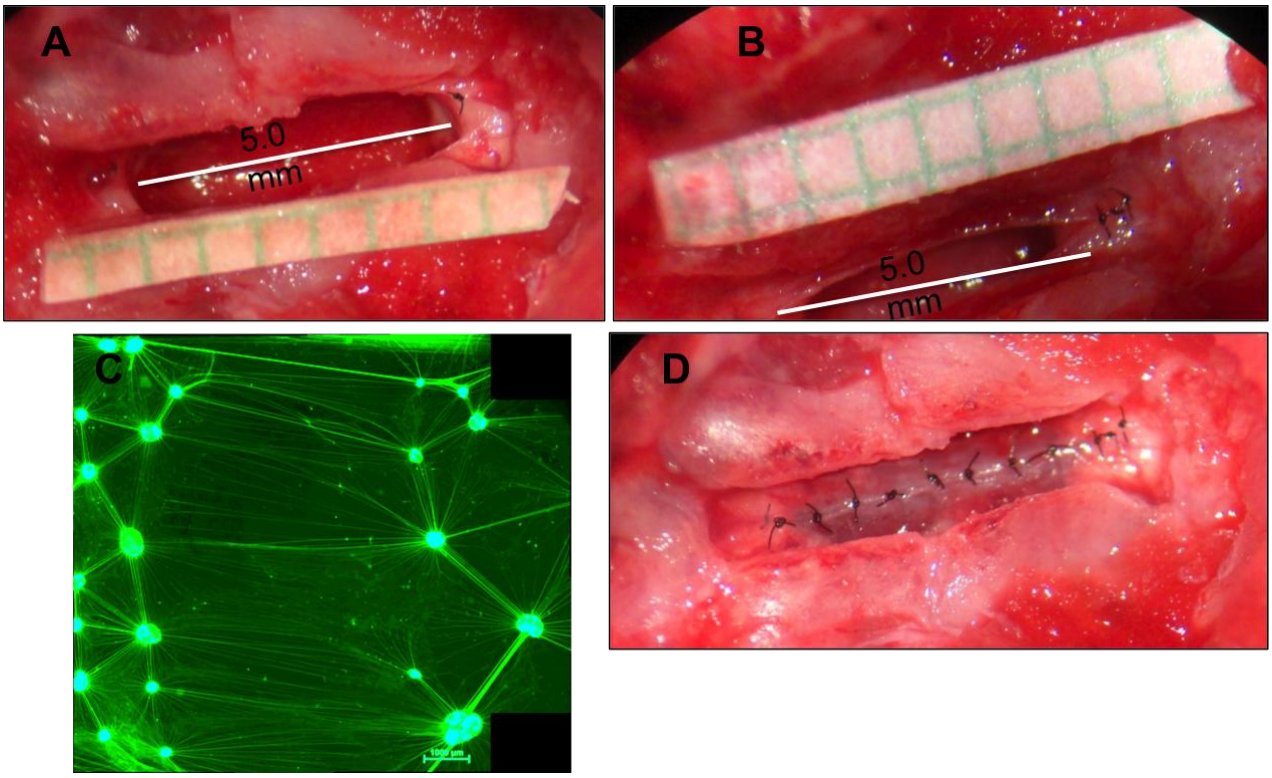

Figure 1: Rodent Spinal Cord Injury Model. (A) The initial spinal cord injury consists of a 5.0 mm transection and evacuation of the cord at the levels of T10-T11. Collagen hydrogel is inserted into the cavity. **(B)** At 9-10 days post-initial injury, the cavity is reopened, the hydrogel is removed, and a 5.0 mm gap exists. **(C)** GFP+ TENGs (5.0 mm in length) stretch grown in culture are encapsulated in a collagen type I hydrogel and rolled into the cavity site, bridging the spinal cord injury. **(D)** Post injury and replacement with either a TENG or a collagen hydrogel control, the dura is sutured together for protection and prevention of invading tissue.

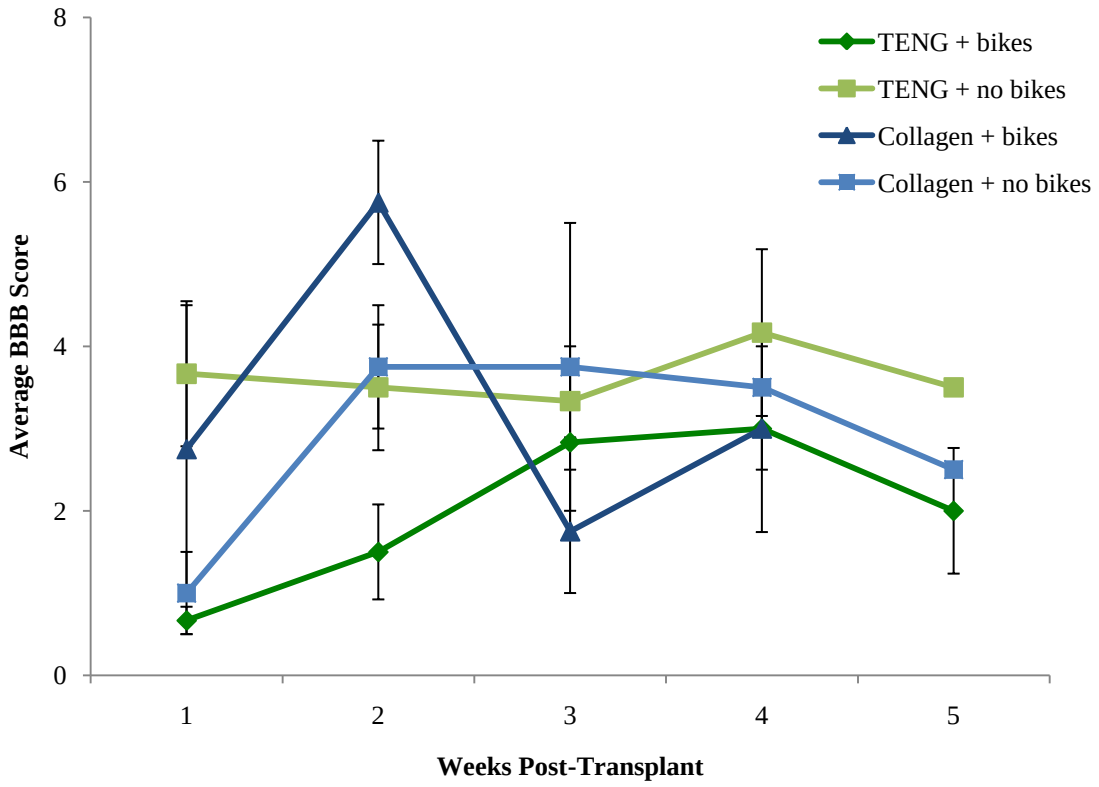

Figure 2: Average Scores on the Basso, Beattie, and Bresnahan (BBB) Scale. Scores among animals that received transplants of tissue engineered nerve grafts embedded in a collagen hydrogel (TENG) or the collagen hydrogel only (collagen), with and without bike passive rehabilitation three times weekly (bikes). All animals scored 21 (highest points possible) on the scale prior to spinal cord injury. TENG + bikes: n=3; TENG + no bikes: n=3; collagen + bikes: n=2; collagen + no bikes: n=2. Errors bars indicate standard error.

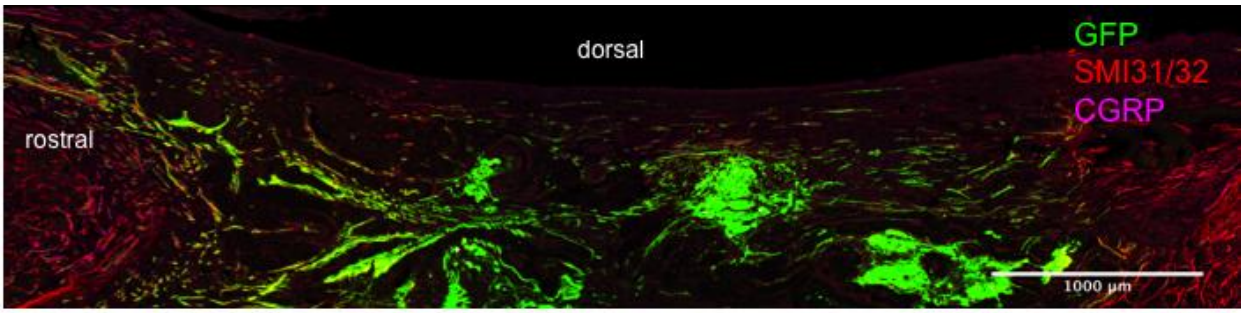

Figure 3: Ten-Day Survival of DRG Nerve Construct Transduced with GFP. Rat TENGs (green) survived (absent immunosuppression) and maintained their morphology both in neuronal bodies and axonal architecture at 10 days post transplant. Rodent SCI (5.0 mm lesion, 10 days post-transplant). SMI31/32 (red) antibody immunostains all axons, both thick and thin. CGRP (Calcitonin gene related peptide) (purple) antibody identifies sensory neurons only.

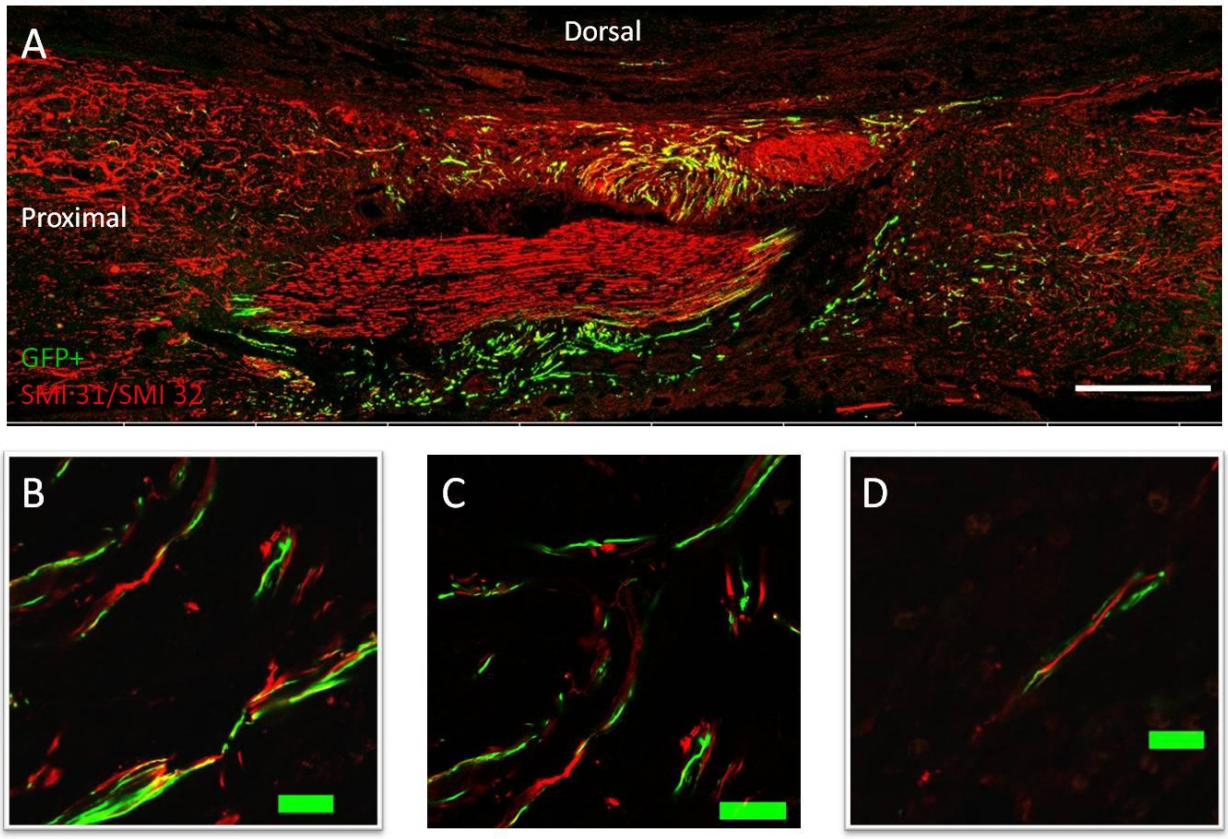

Figure 4: Preliminary Evidence of DRG Nerve Construct Integration with Host Axons. (A) GFP_ TENG (green) at 10 days post-transplantation. SMI 31/32 (red) identifies both thick and thin axons. Scale bar: 1000µm **(B-D)** Magnifications (20X) of (A) showing possible integration of construct with host axons. Immunohistochemical analysis demonstrates preliminary evidence of axon-induced axon regeneration showing host axons (red) growing directly along GFP-positive TENG axons (green) (B,C,&D). The use of GFP-positive green rats with mCherry red TENGs will provide confirmation of this finding. Rodent SCI (5.0 mm, 10 days post-transplant)

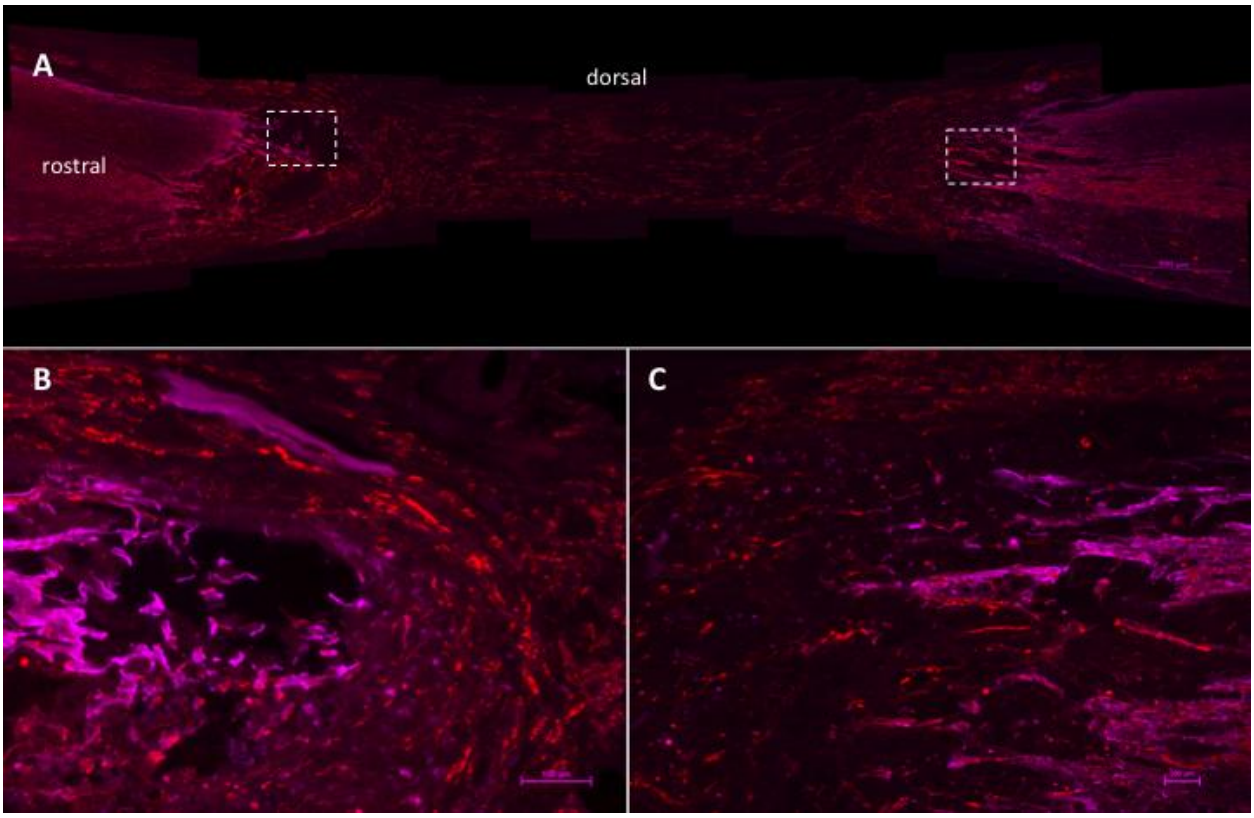

Figure 5: Collagen Only Controls at 1 Month. (A) Collagen only at 1 month post-transplantation. SMI31/32 (red) and GFAP (Glial fibrillary acidic protein) (purple). Image shows evidence of infiltration into collagen hydrogel on both the rostral **(B)** and caudal **(C)** ends of the injury site.

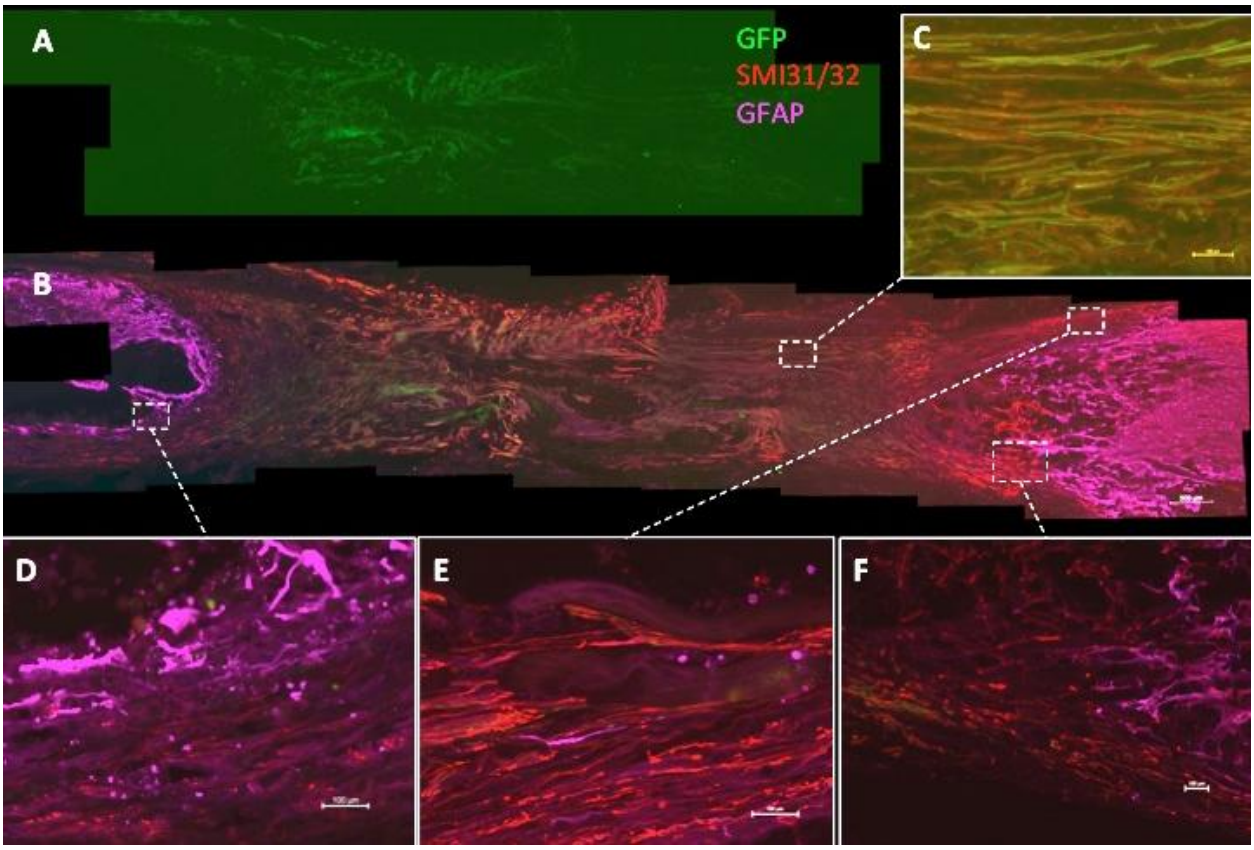

Figure 6. Possible Host Axon Growth Along TENG Axons. (A) GFP+ TENG (green) at 6 weeks post-transplantation. (B) Immunohistochemistry on TENG at 6 weeks post-transplantation showing GFP TENG (green), SMI31/32 axons (red) and GFAP (glial fibrillary acidic protein) (purple). **(C-F)** Magnifications of (B). **(C)** Axon regeneration in TENGs often occurred directly along TENG axons showing possible integration of construct with host axons. Immunohistochemical analysis demonstrates evidence of axon-induced axon regeneration showing host axons (red only) growing directly along GFP-positive TENG axons (green). The use of GFP-positive green rats with mCherry red TENGs will provide confirmation of this finding. **(D)** Rostral end of injury site shows strong GFAP response, indicating possible glial scar formation. **(E,F)** Caudal end of injury site shows axons positive for SMI31/32 possibly growing into the TENG axons. Rodent SCI (5.0 mm, 6 weeks post-transplant).

Challenges and Future Directions:

As mentioned previously, future studies will employ GFP+ host animals receiving mCherry red transduced TENGs to identify host vs graft axons. Tract tracing will also be performed to provide further evidence of host growth into, along, and past TENGs. For tracing, bilateral injections of FluoroEmerald and FluoroRuby dyes into the hindlimb motor cortex and red nucleus will be performed. This will allow for the labeling of corticospinal and rubrospinal descending tracts in the spinal cord, respectively. These tracer injections will be performed 1 week prior to sacrifice to allow for enough time to transport down the spinal cord.

Additionally, we will continue to perform behavioral testing via BBB scoring to identify any functional differences between treated animals. Currently we do not see any differences in animals treated with or without bike rehabilitation but based on previous data from other groups using this technique it is too early yet to see differences; we should not expect any differences until 8 weeks post-transplant. Also, literature shows that there is strong evidence of activity dependent plasticity within the spinal cord after exercise. Therefore, we will continue to perform the bike rehabilitation in order to determine if enhanced spinal cord plasticity will translate into greater behavioral recovery.

KEY RESEARCH ACCOMPLISHMENTS

Project Specific Aim #1:

- BBB: No significant differences have been observed between experimental groups. Current range of scores indicates some hindlimb joint movement but no coordinated movements or plantar placement. Final measures of functional recovery will be measured at 3 months post-transplant.
- In addition to BBB testing for evaluation of functional recovery, we plan to include in vivo tracing of neural tracts in all experimental groups at the final time point in order to evaluate axonal reconnections post-transplant.

Project Specific Aim #2:

- Demonstrated survival and maintenance of axonal architecture of TENGs at 10 days (Figure 3) and up to 6 weeks post-transplant (Figure 6), without immunosuppression.
- Observed preliminary evidence of axon-induced axon regeneration across TENGs in as little as 10 days (Figure 3) and out to 6 weeks post-transplant (Figure 6). If proven true, this finding would confirm findings our group has found with TENGs in peripheral nerve models of repair.
- Future studies are planned to have mCherry red TENGs transplanted into GFP-positive host rats in order to discriminate between host (green) and graft (red) tissue. This will allow us to definitively differentiate between host and graft axons and therefore confirm host axonal regeneration into, across, and out of our TENGs at the injury site.
- Current studies are progressing on schedule, as we have enrolled 19 out of 24 animals thus far:
    - Hydrogel only 4 weeks: n=5
    - TENG 4 weeks: n=4
    - Hydrogel only 3 months: n=4
    - TENG3 months: n=6
- Following completion of the above study, we plan to enroll 8 additional GFP+ animals to more easily identify host regeneration across TENGs. For this future study, all TENGs will be virally transduced to express mCherry red instead of GFP. 2 of the 8 animals will be collagen only and 6 will receive TENGs. These animals will not receive passive rehabilitation and will be sacrificed at an early timepoint (4 weeks) as well as a later timepoint (12 weeks).

REPORTABLE OUTCOMES

Due to our strong efforts this year aimed at incorporating functional assessment and bike rehabilitation into our model, we have no published reports as of yet. As mentioned previously, we have almost completed our complete study of 24 animals and have future plans to begin a second study just after to confirm host vs. graft tissue. We anticipate reporting histological results shortly demonstrating long term survival of our constructs (3 months) and potentially the penetration of host axons through our constructs to form new intraspinal circuits. Tract tracing experiments will also be performed at time of sacrifice to further corroborate results.

CONCLUSION

Over the course of this third year of funding, we have evaluated our TENGs in a 5.0 mm SCI model and have found robust survival and evidence of nerve regeneration across our TENGs at 6 weeks post-transplantation. One of the obstacles we encountered in our study is distinguishing host from transplanted tissue. We are currently addressing this issue by using histological identification of host vs. graft and have implemented additional GFP-transgenic rats to facilitate identification. Success of our studies will provide another avenue to bridging extensive SCI lesions to restore function. TENGs in this study also demonstrated preliminary results indicating axon-induced axon regeneration, a phenomenon we believe is not possible with a collagen hydrogel treatment only. Although our study in ongoing, results collected thus far support premise that TENGs may enable regeneration across nerve lesions following SCI.

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